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In situ detection of warfarin using time-correlated single-photon counting

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ABSTRACT

Here we report on a novel method for the direct *in situ* measurement of specific isomeric forms of the anticoagulant warfarin using time correlated single-photon counting (TCSPC) spectroscopy in conjunction with synthetic Sudlow I binding site receptors. The method is highly robust over the clinically significant concentration range, and demonstrates the potential of the binding site mimics in conjunction with the spectroscopic strategy employed here for the determination of this important pharmaceutical in clinical or even environmental samples.

1. Introduction

The coumarin derivative warfarin is widely used as an anticoagulant drug in the treatment and prevention of thrombolic disorders such as myocardial infarction and stroke [1]. Due to the narrow therapeutic window of warfarin, variability in dose–response and interactions with other compounds, careful monitoring of the effect of drug dosage must be performed [2]. Despite its widespread use and the difficulties in adapting dosage to clinical function, no assay involving the direct detection of this anticoagulant is currently used in general clinical practice. In principle, the fluorophoric nature of coumarins should allow for a fluorescence spectroscopy-based monitoring of warfarin. Recent efforts by us [3–5] using a series of theoretical and spectroscopic studies have highlighted the complex nature of warfarin, and in particular the medium dependent isomerization of this drug (see Chart 1), perhaps illustrating why spectroscopy-based methods for the direct determination of warfarin have not been forthcoming. The unraveling of the relationship between molecular environment, isomeric distribution and the spectroscopic characteristics of the coumarin derivative has afforded us the possibility for developing detection methods, which can potentially discriminate between warfarin in various states, e.g. bound to protein [3], polymeric synthetic receptors [6], and as a general molecular probe [7].

Here we combine the recognition characteristics of a polymeric synthetic receptor system, a human serum albumin Sudlow I binding site mimic [6], with warfarin's molecular environment-sensitive fluorescence emission signal [3] to provide a basis for a robust technique for warfarin determination. The detection strategy described here could have impact for both clinical and even environmental monitoring [8].

2. Materials and methods

2.1. Chemicals

Racemic warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin, min. 98%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). All solvents were of analytical grade and used as received.

2.2. Fluorescence spectroscopy

The warfarin imprinted (MIP) and the corresponding reference (REF) methacrylic acid–ethylene dimethacrylate co-polymers used were prepared as previously reported [6].

Prior to fluorescence measurements, polymer particles were allowed to swell for at least 20 h in acetonitrile (5 mg mL^{-1}). All fluorescence spectroscopic measurements were typically performed

Abbreviations: TCSPC, time correlated single photon counting; MIP, molecularly imprinted polymer; REF, reference polymer.

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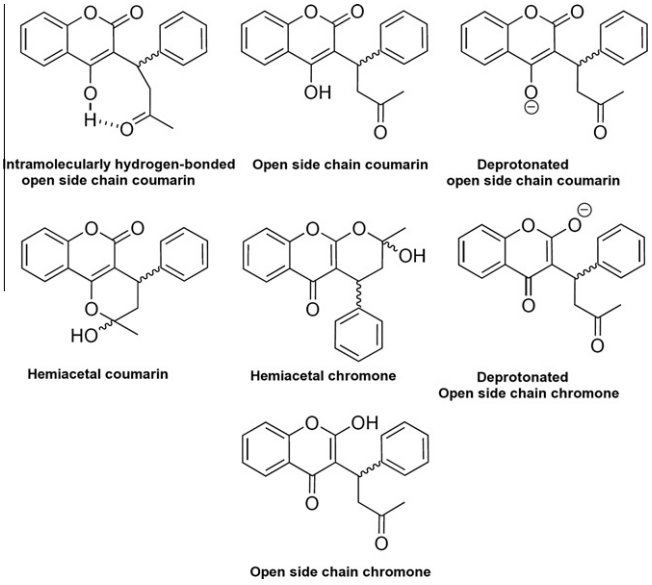

Chart 1. Possible solution structures of warfarin previously reported in the literature [13,14].

under continuous stirring using a standard quartz-cuvette (1 cm path length) and a polymer concentration of 0.5 mg mL^{-1} (total volume 2 mL) at room temperature, unless otherwise stated.

2.3. Steady state

Fluorescence steady state experiments were performed on a Fluoromax-2 spectrofluorometer (ISA Jobin Yvon-SPEX, USA). Fluorescence emission spectra were typically recorded between 320 and 500 nm with an excitation wavelength set to 295 nm. Emission spectra were corrected for the wavelength dependence of the detection system and Xe lamp (150 W) fluctuations. To reduce the impact of light scattered by the polymer particles, a cut-off filter of 389 nm was placed in front of the emission monochromator slit with a 1 nm spectral bandwidth. The emission spectrum of warfarin in acetonitrile demonstrated a maximum at 410 nm. The fluorescence spectral form was shown to be independent of excitation wavelength, i.e. 295, 305 and 330 nm, which ensured the purity of the sample. The excitation monochromator spectral bandwidth was set to 5 nm. Upon recording spectra the increment step was 1 nm and the integration time 0.5 s. In the intensity based analysis of steady state spectra the background emission from polymer solution itself was always subtracted from warfarin containing polymer solution.

Time-based scans were used to estimate the time required to equilibrate the binding of warfarin to the polymers in acetonitrile. Fluorescence intensity measurements were started directly after the addition of warfarin (final concentration in a cuvette was either 0.5 or 2 μM) to the polymer solutions. In these experiments the sample fluorescence signal was measured with excitation at 295 nm and emission detection at 440 nm. The time increment employed was 1 min, with an illumination time of 5 s. To avoid photo bleaching the shutter was closed between each excitation period.

In a subsequent series of steady state titration experiments, different amounts of polymer (0.02–2.0 mg mL^{-1}) were incubated with a constant concentration of warfarin (2 μM) for 3 h. The binding of warfarin to the different amounts of polymer was measured as a function of changes in the fluorescence intensity at 440 nm. In order to measure the background fluorescence of the polymers studied control experiments were performed using the same polymer concentrations as mentioned above, though in the absence of warfarin.

2.4. TCSPC

Time resolved fluorescence spectroscopic measurements were performed on a time correlated single-photon counting (TCSPC) system, IBH 5000 M (Jobin Yvon IBH Ltd., Glasgow, UK) equipped with IBH reconvolution software. The decay times with associated amplitudes (A_i) were determined using a light emitting diode, NanoLED-17 (HORIBA Jobin Yvon IBH) producing 295 nm excitation pulses at 1.0 MHz repetition rates. A single grating monochromator (Model 5000M IBH) with a spectral bandwidth set to 32 nm together with a cut-off filter of 389 nm was applied to eliminate scattered light from sample polymer particles. The data were collected in 4048 channels and the time-calibration was 13.4 ps/channel. The fluorescence emission was detected by an IBH TBX-04 photon detection module under TCSPC conditions, and the full width at half maximum (fwhm) of the instrumental response function was typically around 560 ps, which was measured with a suspension of silica particles (Ludox TMA-34 Sigma–Aldrich) dissolved in deionized water. All experiments were performed monitoring the kinetics at 440 nm at an angle of 90° relative the excitation light. Time resolved fluorescence data were analyzed using IBH DAS6 decay analysis software, which functions upon least-squares fitting algorithms and reconvolution with the experimental response function. Three decay times were generally needed to obtain satisfying fitting results, i.e. $\chi^2 \leqslant 1.2$. The fitting results from the time (t) dependency were presented as amplitudes (A_i) and decay times (τ_i) in relation to Eq. (1):

$$F(t) = A_0 + A_1 e^{-\frac{t}{\tau_1}} + A_2 e^{-\frac{t}{\tau_2}} + A_3 e^{-\frac{t}{\tau_3}}. \quad (1)$$

All values reported are presented as mean ± standard deviation if not otherwise stated.

3. Results and discussion

It is envisaged that the combination of a warfarin molecularly imprinted polymer (MIP) [9,10] mimic of the human serum albumin Sudlow I binding site [6] with warfarin's molecular environment-sensitive fluorescence emission signal [3] can provide the basis for a robust technique for warfarin determination. It is important to note that while the warfarin–MIP binding studies did not demonstrate evidence of specific binding when using acetonitrile as a rebinding medium, closer examination using time resolved fluorescence detection made it possible to monitor the binding of warfarin to the MIP particles *in situ* [7]. Analysis of the binding of warfarin to the MIP, as well as to the reference polymer (REF), in acetonitrile revealed lifetimes indicative for the deprotonated open side chain form of warfarin free in solution, but for the MIP a long component of about 5 ns was also detected, Tables 1 and 2. Importantly, it is probable that this longer lifetime originates from a stabilized structure of warfarin bound to the polymer matrix. Collecting the amplitude of the longer lifetime, A_3, at various concentrations of polymer it became apparent that this amplitude was correlated to the stoichiometry between the amounts of MIP and warfarin present. By monitoring the amplitude associated with the bound state of warfarin, A_3, a saturation binding curve could be constructed in which the polymer concentration required for 50% binding of warfarin was 0.58 ± 0.10 mg mL^{-1} (R^2 = 0.95, Supplementary Fig. S3). The sensitivity of the technique permits the use of small amounts of polymer, which in itself is advantageous as this reduces light scattering. Using a similar approach, maintaining the polymer concentration constant, the

Table 1
Typical fit parameters for the fluorescence decay profile of a constant concentration of warfarin in the presence of different concentrations of MIP in acetonitrile.[a]

	Lifetimes (ns)			Amplitudes (%)		
[MIP] (mg mL^{-1})	τ_1	τ_2	τ_3	A_1	A_2	A_3
0.00[b]	0.31	1.51		4.00	96.0	
0.05	0.26 ± 0.04	1.51 ± 0.02	5.09 ± 0.27	4.0 ± 0.5	93.3 ± 0.8	2.7 ± 0.3
0.10	0.18 ± 0.05	1.48 ± 0.02	4.29 ± 0.7	3.4 ± 0.5	93.4 ± 0.7	3.2 ± 1.1
0.20	0.45	1.53	5.26	5.8	85.9	8.3
0.25	0.41 ± 0.05	1.52 ± 0.02	5.40 ± 0.50	6.2 ± 0.2	84.0 ± 3.2	9.8 ± 2.9
0.50	0.38 ± 0.07	1.54 ± 0.02	4.89 ± 0.07	5.3 ± 0.8	75.7 ± 1.6	19.1 ± 2.3
0.75	0.54 ± 0.06	1.61 ± 0.04	5.06 ± 0.07	8.2 ± 1.4	68.4 ± 4.4	23.4 ± 3.3
1.00	0.47 ± 0.05	1.54 ± 0.01	4.90 ± 0.02	7.2 ± 2.2	70.9 ± 2.5	21.9 ± 2.2
1.50	0.57	1.56	4.91	11.8	58.2	30.0
2.00	0.72	1.75	5.32	13.8	61.5	24.7

[a] λ_{exc} = 295 nm, λ_{em} = 440 nm, [warfarin] = 0.5 μM (this concentration was chosen after a series of steady state fluorescence spectroscopy experiments, Supplementary Table S1 and Fig. S1). Values above are presented as mean ± standard error of the mean with $n \geq 3$ if possible.
[b] [Warfarin] = 2.0 μM.

Table 2
Fit parameters for the fluorescence decay profile of different concentrations of warfarin in the presence of a constant concentration of MIP in acetonitrile.[a]

[Warfarin] (μM)	Lifetimes (ns)			Amplitudes (%)		
	τ_1	τ_2	τ_3	A_1	A_2	A_3
0.00	0.52 ± 0.09	2.79 ± 0.04	5.45 ± 0.17	6.3 ± 0.4	35.7 ± 3.0	58.1 ± 3.3
0.25	0.42 ± 0.05	1.57 ± 0.02	4.91 ± 0.08	5.3 ± 0.8	70.5 ± 2.6	24.1 ± 2.8
0.50	0.40 ± 0.07	1.55 ± 0.02	4.95 ± 0.07	5.3 ± 0.8	72.7 ± 2.7	22.1 ± 2.9
0.75	0.36 ± 0.05	1.52 ± 0.02	4.89 ± 0.08	4.7 ± 0.8	83.0 ± 1.9	12.3 ± 1.9
1.00	0.37 ± 0.06	1.53 ± 0.02	4.98 ± 0.12	4.9 ± 0.8	85.0 ± 0.8	10.1 ± 0.1
2.00	0.33 ± 0.04	1.52 ± 0.01	4.93 ± 0.08	4.6 ± 0.5	88.7 ± 1.1	6.8 ± 1.1
5.00	0.40 ± 0.02	1.53 ± 0.00	4.93 ± 0.10	5.5 ± 0.3	90.9 ± 0.3	3.6 ± 0.1

[a] λ_{exc} = 295 nm, λ_{em} = 440 nm, [MIP] = 0.5 mg mL^{-1} (this concentration was choosen after a series of steady state fluorescence spectroscopy experiments, Supplementary Fig. S2). Values above are presented as mean ± standard errors of the mean with $n \geqslant 3$.

MIP binding affinity for warfarin was investigated which allowed the determination of an apparent K_D of 0.56 ± 0.09 μM (R^2 = 0.93, Supplementary Fig. S4). Importantly, this concentration range coincides with the clinically therapeutic window [11,12]. It is important to note, that the TCSPC technique allowed the clear differentiation of the bound and unbound states of warfarin in acetonitrile.

In summary, the results arising from the interrogation of warfarin – synthetic polymer Sudlow I binding site mimic binding events by TCSPC fluorescence spectroscopy demonstrates the potential for combining the binding site mimics and the spectroscopic strategy employed here for the determination of warfarin; and highlights the possibility for the development of sensor devices for use in the clinical and environmental monitoring of this important and widely used pharmaceutical.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.103.

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